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Evidence for presence of the founder Ia mtDNA haplotype of *Phytophthora infestans* in 19th century potato tubers from the Rothamsted archives

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Late blight remained a significant disease for potato growers in Europe long after the famine of the 1840s. Of the four mitochondrial haplotypes of *Phytophthora infestans*, only the Ia mitochondrial DNA (mtDNA) haplotype has been identified previously in infected potato leaves from famine-era herbarium specimens collected in England, Ireland and Europe in the 19th century. Long-term soil fertility experiments were conducted on potato between 1876 and 1901 in Rothamsted to investigate effects of combinations of organic manures and mineral fertilizers on disease and yield. This report identifies for the first time the same Ia mtDNA haplotype of *P. infestans* in three diseased tubers from 1877 from the long-term Rothamsted trials, thus providing the earliest evidence of the presence of the founder Ia mtDNA haplotype of *P. infestans* in potato tubers in England. Soil amendments had a significant impact on disease and yield. A real-time PCR assay was used to detect and quantify *P. infestans* in tubers. The level of pathogen DNA was greatest in tubers from highest yielding plots that received combinations of inorganic nitrogenous and mineral fertilizers and least in tubers from plots with organic farmyard manures or non-nitrogenous mineral fertilizers. The Ia mtDNA haplotype was also confirmed from diseased potato leaves during the same time period. Thus, the founder Ia mtDNA haplotype survived in potato tubers after 1846 and was present over 30 years later in the UK.

Keywords: late blight, oomycetes, *Phytophthora infestans*, potato famine

Introduction

Phytophthora infestans causes late blight of potato, one of the world's most devastating plant diseases, and was responsible for the Irish potato famine. Epidemics began by introduction of the pathogen on potato tubers. The pathogen then spread rapidly first in the US and then European potato crops by airborne inoculum. Speculation about the cause, origin, source of inoculum and methods to control potato late blight began soon after Ireland's potato famine in the 1840s (Berkeley, 1846). It was first hypothesized that epidemics originated from tubers imported from South America (Berkeley, 1846; Bourke, 1964). The Ia mtDNA haplotype of *P. infestans* was identified in infected potato leaves from 19th century herbarium specimens from the UK and Europe (May & Ristaino, 2004). Genotypic analysis of modern day populations suggests an Andean source for the 19th century UK and European epidemics (Ristaino *et al.*, 2001; Gomez-Alpizar *et al.*, 2007). Other investigators suggested that tubers imported from Mexico infected with *P. infestans* clonal lineage US-1 (mtDNA haplotype Ib) caused famine-era epidemics (Goodwin *et al.*, 1994). The data here do not support the 19th century introduction of the Ib mtDNA haplotype hypothesis, but suggest a mid-20th century introduction for this haplotype (Ristaino & Hu, 2009; Ristaino, 2012). Up until now, there has been no direct evidence of *P. infestans* in 19th century potato tubers or evidence for survival of the founding Ia mtDNA haplotype by tuber-borne inoculum.

After another severe outbreak of potato late blight in Europe in 1872, a special committee of the Council of the Royal Agricultural Society of England began new investigations into the disease (Gilbert, 1888, 1900; Warren & Johnston, 1960). By 1876, scientists had clearly attributed the disease to the causal agent *P. infestans* (de Bary, 1876) and besides developing resistant varieties, there was interest in studying the effect of soil fertility amendments on severity of tuber blight (Gilbert, 1888). John Bennet Lawes and Joseph Henry Gilbert (Fig. 1a,b) started a long-term field experiment at Rothamsted to examine effects of manuring, mineral fertilization, and host resistance on the severity of epidemics and yield of potatoes (Gilbert, 1888, 1900; Warren & Johnston, 1960). The experiment consisted of continuously cropped potatoes from 1876 to 1901 on the Hoosfield site (Gilbert, 1888; Hall, 1905). Lawes and Gilbert had the foresight to archive dried ground potato tubers from this experiment (Fig. 1c). The Rothamsted archive now contains a unique collection of 200 000 samples of plant material and soils taken since 1843 from a series of long-term experiments whose history is fully documented. Samples from this archive have been used previously to examine the long-term abundance of two wheat pathogens and the impact of air pollutants on disease occurrence (Bearchell *et al.*, 2005).

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Figure 1 Rothamsted Research was the site of the earliest experiments to determine the effect of soil fertility on severity of late blight of potatoes. Portraits of (a) John Bennet Lawes and (b) Joseph Henry Gilbert, who published a paper 'Results of Experiments at Rothamsted on Growth of Potatoes: Twelve Years in Succession on the same land' in 1888 (Gilbert, 1888). Soil fertility amendments were applied and severity of potato blight was recorded. (c) Harvested potato tubers (dried, ground and placed in glass bottles) from the Hoosfield experiment are located in the Rothamsted archive. Samples of tubers were labelled 'good', 'small', 'bad' or 'diseased'.

The objectives were to address the following questions: (i) can *P. infestans* DNA be detected and quantified in the diseased ground potato tubers from the Hoosfield experiment; (ii) can the mtDNA haplotype be determined; and (iii) what are the relationships between the amount of *P. infestans* DNA in tubers, soil fertility treatment, recorded disease and yield.

Materials and methods

Samples

After winter wheat given various fertilizer treatments, potatoes were grown on the Hoosfield site between 1856 and 1875. The susceptible potato cultivar Rock was planted in years 1876 to 1879 and was the principal potato variety grown in the UK after the famine. The cultivar Champion was planted from 1880 to 1901 in the plots. This potato cultivar had high levels of resistance to tuber and foliar blight (Davidson, 1933). There were 10 soil fertility treatments applied to the potatoes (Table 1). Plots were 0·07 ha in size and a single row of potatoes was planted in each plot to a depth of 12 cm. The entire experiment was 0·85 ha and the 10 treatments were not replicated within the trial. Complete records of potato yields, weather conditions and disease were made (Gilbert, 1900; Hall, 1905). Each year at harvest in September/October from 1876 to 1879, potato tubers were evaluated for disease, divided into good, small or bad (diseased) and stored in the archives (Fig. 1c). The long-term experiments continued from 1876 to 1901 and potatoes were harvested each year and a subsample of several tubers were dried, ground, placed in glass bottles with cork stoppers and stored in the Rothamsted archive.

Samples (2 g) of dried ground tubers (composite dried samples from each plot) stored in the Rothamsted archive between 1876 and 1879 were removed aseptically from each jar and placed in paper coin envelopes. A subsample (30 mg, $n = 61$) from each was placed in sterile plastic tubes for subsequent work. Initial work was done with 19 samples labelled as bad or diseased that were taken from specimens harvested over a 4-year period (1876–1879) (Table 1; Fig. 1). The rest of the samples were labelled good or small. Visible darkening of the ground diseased tubers was apparent. Plots were planted with susceptible cv. Rock from 1876–1879. No potatoes labelled diseased were present among the archival samples after 1880 when the 'blight resistant' cv. Champion was planted in the plots (Davidson, 1933; Glendinning, 1983). Recorded disease severity in tubers was less after 1880, and only potato samples labelled as good were archived after 1879 (Gilbert, 1888, 1900), so samples of these tubers were not tested with conventional or quantitative PCR.

Potato leaf samples infected with *P. infestans* were also collected between 1879 and 1886 in the UK and Ireland and were stored in herbaria collections at the Royal Botanic Gardens Kew, UK, and the Dublin Botanic Gardens, Glasnevin, Ireland (Table 3; May & Ristaino, 2004). Ten milligram samples were removed from infected leaf lesions and placed in sterile plastic tubes.

Table 1 Average yield (t ha^{-1}) of potatoes[a] in experiments to study the effects of fertilizer treatment on yield and severity of late blight disease caused by *Phytophthora infestans* at Rothamsted, Harpenden, UK for the period from 1876 to 1879

		Yield (t ha^{-1})				
Plot	Treatment[b]	Good	Small	Diseased	Total	% diseased[c]
1	Nil (control)	5·51	0·78	0·35	6·64	5·3
5	N	5·92	0·83	0·54	7·29	7·4
6	N*	7·78	0·75	0·85	9·37	9·1
9	P	7·37	1·04	0·63	9·03	7·0
10	P, K, Na, Mg	8·01	0·82	0·66	9·49	7·0
7	N, P, K, Na, Mg	14·30	1·11	1·92	17·33	11·1
8	N*, P, K, Na, Mg	14·93	1·01	2·35	18·29	12·8
2	FYM	9·52	1·00	0·88	11·40	7·7
3	FYM, P	10·68	1·08	1·04	12·79	8·1
4	FYM, N*, P	13·67	0·93	2·38	16·99	14·0

[a]At harvest, in September/October, potatoes were separated into good, small and diseased tubers. Cultivar Rock was grown from 1876 to 1879.
[b]Fertilizer rates: N (96 kg ha^{-1}) ammonium sulphate (224 kg ha^{-1}) + ammonium chloride (224 kg ha^{-1}); N*(96 kg ha^{-1}) sodium nitrate (616 kg ha^{-1}); P (33·5 kg ha^{-1}) superphosphate (440 kg ha^{-1}), made from bone ash (224 kg), sulphuric acid (168 kg) and water; K (135 kg ha^{-1}) potassium sulphate (336 kg ha^{-1}); Na (16 kg ha^{-1}) sodium sulphate (112 kg ha^{-1}); Mg (11 kg ha^{-1}) magnesium sulphate (112 kg ha^{-1}); FYM (224 kg N ha^{-1}) farmyard manure (35 t ha^{-1}).
[c]Weight diseased/total weight tubers harvested × 100; the proportion of tubers diseased is likely to have been underestimated because some diseased tubers may have rotted before harvest.

DNA extraction

All work was done in a laboratory without any work with modern *P. infestans*. DNA was extracted with either a modified CTAB method or with a DNeasy Plant Mini Kit (QIAGEN) at Rothamsted Research or the North Carolina State University Phytotron containment lab that was equipped with supplies, reagents and separate equipment from the lab where modern late blight research was done (Trout *et al.*, 1996).

Quantitative TaqMan PCR for *P. infestans*

A quantitative PCR assay was developed using the ribosomal internal transcribed spacer 2 (ITS2) region (Trout *et al.*, 1996; Ristaino *et al.*, 2001). A TaqMan probe (Pinf: 6-FAM-5′-CGAGTTGGC GACCGG-3′-MGB-NFQ) and primer sets (PinfRT-F: 5′-CTG GTTGTGGACGCTGCTATT-3′ and PinfRT-R: 5′-TTAACGCCG CAGCAGACA-3′) were designed with PRIMER EXPRESS v. 2.0 (Applied Biosystems). The specificity of the primer probe combination was tested against *P. infestans* and a series of *Phytophthora* species known to infect potato (*P. cactorum*, *P. cryptogea*, *P. drechsleri*, *P. erythroseptica*, *P. megasperma* and *P. nicotianae*). First, a series of experiments was done with four primer–probe combinations (900/250 nM, 450/125 nM, 225/62·5 nM and 112·5/31·25 nM of primer/probe, respectively) and a range of DNA concentrations. The PCR was optimized using the 450 nM primer/125 nM probe concentrations and a standard curve was done using known levels of pathogen DNA (10 ng, 1 ng, 100 pg, 10 pg, 1 pg and 100 fg) for each experiment to relate C_t values to the log of DNA concentration (pg mg^{-1}; Fig. 2a). The lowest threshold level for sensitivity was determined. A series of quantitative TaqMan PCR experiments was also done using *P. infestans* DNA extracted from mycelium (12·5, 25 and 50 ng) and DNA extracted from modern leaves infected with *P. infestans* (1, 1·5 and 2 μL; Fig. 2b). Controls consisted of DNA extracted from healthy potato leaves and no template DNA. Quantitative PCR was done on all 61 samples from the archives collected from 1876 to 1879. All work with archival material was done in the Phytotron containment lab separate from the work with modern DNA.

The 20 μL reaction mixture contained 1 × TaqMan Universal PCR Master mix without AmpErase UNG, 0·45 μM each primer and 0·125 μM TaqMan probe, and 1 μL DNA extracted from either a mycelial culture or dried potato tubers. The thermal cycling conditions consisted of a 50°C UNG activation step for 2 min, 95°C for 10 min, and 40 cycles of 95°C denaturation for 15 s and 60°C anneal/extension for 1 min in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). The threshold value was set at a fluorescence (ΔRn) of 0·05. A standard curve was done using known levels of pathogen DNA (10 ng–10 pg) for each experiment (Fig. 2a). A cycle threshold (C_t) value <35 was scored as a positive result.

Conventional PCR with ITS and mtDNA primers

Conventional PCR was done with *P. infestans* specific primers PINF2 and Herb2 (Herb2: 5′-CGGACCGACTGCGAGTCC-3′; PINF2: 5′-CTCGCTACAATAGCAGCGTC-3′) on 19 samples (Table 2) of tubers labelled bad or diseased (Ristaino *et al.*, 2001). These primers amplify an approximately 100 bp region of the internal transcribed spacer (ITS) region 2. The spacer region 2 of ITS DNA is not polymorphic within *P. infestans* but it is diagnostic for *P. infestans* and was used to confirm the presence of amplifiable *P. infestans* DNA in the samples (Ristaino *et al.*, 2001). Each DNA extraction was done twice and DNA was sequenced to confirm sequences in the forward and reverse directions. DNA that was amplified was sequenced on an ABI Prism System automated sequencer (Model 377, v. 2.11, Perkin Elmer).

Mitochondrial primers that amplify restriction sites diagnostic for the four mitochondrial haplotypes were used (Griffith & Shaw, 1998; Ristaino *et al.*, 2001; Avila-Adame *et al.*, 2005). Primer pairs P3F1/P3R1 amplify a 195 bp region of DNA near the 3′ end of *rpl5* gene in the P3 region of the mitochondrial genome. The type I mtDNA haplotypes possess an *Eco*RI site in this region that is absent in the type II mtDNA haplotypes. The primer pairs P4F2/P4R3 amplify a 188 bp region near the 3′ end of the *cox1* gene in the P4 region. These primers also amplify an *Eco*RI site that is present in type I mtDNA haplotypes but absent in the type II mtDNA haplotypes. Use of these two sets of primers allowed sepa-

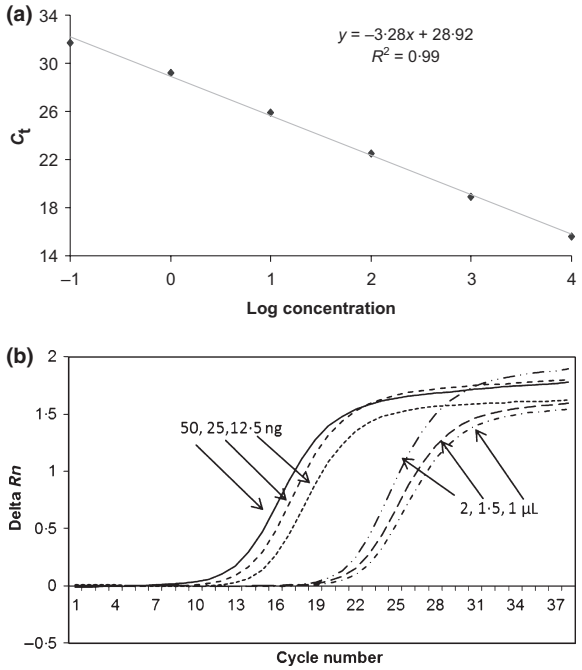

Figure 2 Quantitative TaqMan PCR assay for detection of *Phytophthora infestans*. (a) Relationship between cycle threshold value (C_t) from quantitative PCR and DNA concentration (100 fg to 10 ng) of *Phytophthora infestans* (log_{10}-transformed) for standard DNA samples, assessed by linear regression; (b) quantitative TaqMan PCR amplification curve (ΔRn versus cycle number) for three DNA concentrations (12·5 ng, 25 ng and 50 ng) and three amounts of *P. infestans* DNA extracted from diseased leaves (1 µL, 1·5 µL, 2 µL) at primer/probe concentrations of 450 nM and 125 nM, respectively.

ration of type I from type II mtDNA haplotypes. The primers P2F4/P2R4 amplify a variable region of 167 bp near the 3′ end of the *nad4* gene in the P2 region of the mitochondrial genome (Griffith & Shaw, 1998; Ristaino *et al.*, 2001). Isolates of the Ib mtDNA haplotype contain an *Msp*I restriction site in this region that is not present in the other three modern mtDNA haplotypes. Use of this primer pair in conjunction with either the P3 or P4 primer sets allowed separation of the Ia and Ib mtDNA haplotypes. Primers that amplified the variable restriction sites within *rpl5* (P3), *cox1* (P4) and *nad4* (P2) mt DNA genes were: P3F1: 5′-TTCAAAATGTC TTACAGTTTTTCG-3′/P3R1: 5′- GCAAGGTTATACTCTACC ATTGAGC-3′; P4F2: 5′-GGAATGCTGTAAGTAGTTTTGGTT-3′/P4R3: 5′-TTTAAGATCGTGGTATTAAATTAT-3′; and P2F4: 5′-ACCAATTGTTGCGAAAACAG-3′/P2R4: 5′-TTACGGCGG TTTAGCACAT-3′. All PCR reactions were done twice.

In all PCR reactions, the initial first round PCR products were used as template for a second round of PCR with the same primer pairs. Each DNA extraction was done twice and DNA amplicons were sequenced in the forward and reverse directions to confirm results. PCR amplifications of both ribosomal and mtDNA were done in 50 µL reaction volumes consisting of 2 µL template DNA, 5 µL 10 × PCR buffer (20 mM Tris-HCl, 50 mM KCl, pH 8·4), 34·25 µL sterile distilled water, 2·5 µL 2·0 mM dNTP (100 µM, Invitrogen), 1·8 µL 50 mM $MgCl_2$ (1·8 mM, Invitrogen), 2·5 µL each of a 10 µM primer (0·25 µM), 0·48 µL bovine serum albumin (0·1 mg mL^{-1}) and 0·2 µL 5 U μL^{-1} *Taq* DNA polymerase (1 U, Invitrogen). Positive controls were run in a separate lab with the same master mix and included DNA from a *P. infestans* infected potato tuber or DNA extracted from mycelium of *P. infestans*. Negative controls included a healthy potato tuber or a no template DNA control. Cycling parameters were initial denaturation at 96°C for 90 s, followed by 35 cycles consisting of denaturation at 96°C for 40 s, annealing at 50°C for 1 min for mtDNA (or 54°C for rDNA), and extension at 72°C for 90 s. A final extension at 72°C for 10 min followed. Amplified products were visualized by gel electrophoresis on 1·6% agarose gels containing 0·5 µg mL^{-1} ethidium bromide with 1 × TBE running buffer (0·5 µg mL^{-1} ethidium bromide). DNA that was amplified from specimens was sequenced on an ABI Prism System automated sequencer (model 377, v. 2.11, Perkin Elmer). Conventional PCR was also done using the same primers and techniques described above on a set of pathogen infected potato and tomato leaf samples collected from the same time period in the UK and Ireland that were stored in herbaria collections at the Royal Botanic Gardens Kew, UK and the National Botanic Gardens, Glasnevin, Ireland (Table 3; May & Ristaino, 2004).

Table 2 *Phytophthora infestans* DNA detected by conventional PCR and quantitative PCR in archival potato tuber samples from 1876–1879 from an experiment at the Hoosfield site at Rothamsted, Harpenden, UK

Year	Plot number[a]	rDNA (ITS)[b]	Mean quantity DNA (pg mg^{-1})[c]
1876	8	+	-[d]
1877	3	+	1·0
1877	6	+	2·1
1877	8	+	54·3
1877	9	+	-
1878	1	+	1·6
1878	1	+	0·7
1878	2	+	12·2
1878	7	+	0·5
1878	8	+	1·0
1878	8	+	-
1878	9	+	25·9
1878	9	+	0·3
1878	10	+	-
1879	1	+	18·7
1879	3	+	10·6
1879	6	+	2·6
1879	7	+	44·7
1879	8	+	45·6

[a]Plot numbers associated with different fertility treatments. See Table 1 for description.
[b]PCR was done with primers for detecting *P. infestans* called PINF2 and Herb2. These primers amplify a region of approximately 100 bp in the internal transcribed spacer (ITS) region 2.
[c]Quantitative PCR done with a TaqMan probe to quantify *P. infestans* on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems) in a 20 µL reaction volume containing 10 µL of TaqMan Universal PCR Master Mix, 0·45 µL each of forward and reverse primers (20 µM), 0·5 µL probe (5 µM), 7·6 µL water and 1 µL of DNA extracted from 30 mg of tissue (1 µL DNA mg^{-1} tissue).
[d]Not determined because quantities were less than cycle threshold value of 35.

Analysis of the data

The SEQUENCE DETECTION Software System v. 1.2.3 (Applied Biosystems) was used to analyse the data. The software was used to define plate standards, and negative and unknown values for the samples;

Table 3 DNA of *Phytophthora infestans* detected by conventional PCR from ITS DNA and PCR of three mitochondrial gene regions in 19th century potato and tomato leaf samples from England and Ireland

Date	Collector	Country	Host	Herbarium[a]	PINF2/Herb2[b]	mtDNA haplotype[c]
1875	J. E. Vize	England	*S. tuberosum*	K	+	Ia
1878		England	*S. tuberosum*	DBG	+	Ia
1879	M. J. Berkeley	England	*S. tuberosum*	K	+	Ia
1879	M. J. Berkeley	England	*S. tuberosum*	K	+	Ia
1882	Thompson	Ireland	*S. esculentum*	DBG	+	Ia
1884	W. B. Grove	England	*S. tuberosum*	K	+	Ia
1886	J. E. Vize	England	*S. tuberosum*	K	+	Ia

[a]Herbaria samples from collections at Kew (K) or the Dublin Botanic Gardens Glasnevin (DBG).
[b]PCR was done with primers for detecting *P. infestans* called PINF2 and Herb2. These primers amplify a region of approximately 100 bp in the internal transcribed spacer (ITS) region 2.
[c]Mitochondrial DNA from the P2, P3 and P4 regions was amplified and sequenced using primer pairs described in the text. The mtDNA haplotype named by methods of Griffith & Shaw (1998).

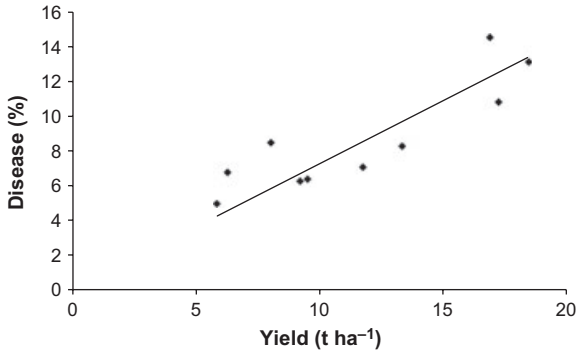

Figure 3 Relationship between proportion of tubers that were diseased (expressed as weight of diseased tubers/total weight × 100) with blight caused by *Phytophthora infestans* (*y*) and yield of potatoes (t ha^{-1}) in Hoosfield experiment (*x*) ($y = 0.73x$, $R^2 = 0.72$).

it also allowed the programming of the thermal cycler reaction settings and once the run was completed, created the amplification plots where C_t values and *Rn* values (measure of reporter signal) could be compared. It was also used to generate standard curves and final values for C_t, quantification, standard deviation of the C_t and mean values for the resulting reactions.

The results of the real-time PCR reactions were visualized by plotting the log of template concentration of DNA against C_t values. PCR efficiency was calculated with the formula $E = (10^{(-1/\text{slope})-1}) \times 100$, where E is the amplification efficiency and the slope is derived from the plot of log of template concentration versus C_t. For example, a slope of 0·73 translated to 95·7% efficiency of amplification (Fig. 2a).

Disease severity, yield and level of DNA

Previous data on yield and disease severity was re-examined in light of the findings on *P. infestans* detection (Gilbert, 1888). Mean disease severity and yield from each of the 10 plots were then averaged over 4 years and are presented in Table 1. A regression analysis was done using mean potato tuber yields and disease severity (Fig. 3). DNA was not detected in tubers from treatments 4, 5 and 10 (Table 2). Regression equations were calculated and R^2 values at the 95% level are shown (Fig. 3).

Results

Soil fertility treatments in the Hoosfield experiment greatly affected the proportion of harvested tubers that were diseased. Disease was less severe in plots that received no fertilizer, or received only farmyard manure or single inorganic minerals or combinations of inorganic minerals without nitrogen (Table 1). The proportion of diseased tubers was greatest in manured plots where additional N was added as sodium nitrate or in plots that received additional N and a mixture of several inorganic minerals (Table 1). The same trends were evident when yields were considered in each year (Table S1). There was a significant positive relationship between diseased tubers and yield (Fig. 3), most likely due to fertility effects on plant growth, tuber bulking, and greater leaf area available for inoculum production.

Quantitative PCR was used to measure the level of *P. infestans* DNA in all 61 of the samples. The DNA amounts are shown for the 19 samples described above and ranged from 0·3 to 54·3 pg mg^{-1} of tissue (Table 2). Higher concentrations of pathogen DNA were found in late blight lesions from dried potato leaves from herbarium samples known to be infected by *P. infestans* (0·72 pg–8·85 ng mg^{-1}). Nineteen tuber samples were positive for PCR. Of these, 15 were positive for *P. infestans* in conventional PCR and also positive by quantitative PCR (Table 2). There was no significant correlation between the level of pathogen DNA in tubers and the percentage of diseased tubers in the whole plots.

DNA was amplified, and ITS DNA was sequenced from 19 samples from 1876 to 1879 that were labelled diseased or bad potato tubers (Fig. 1c). The presence of *P. infestans* in 19 tuber samples was confirmed by conventional PCR amplification with *P. infestans* specific ITS primers (Table 2) and by sequencing (Fig. 4). PCR-amplifiable pathogen DNA was detected in all samples labelled diseased or bad but not in samples labelled good or small.

Mitochondrial DNA from the P2, P3 and P4 regions was also amplified and sequenced from the 19 samples. The Ia haplotype was identified in three diseased tuber samples from plots 6, 8 and 9 from 1877 (Fig. 5). Polymorphisms at

three loci confirmed the identity of the mtDNA haplotype as Ia in the tubers.

Pathogen infected potato leaf samples collected from the same time period in England and Ireland yielded ITS DNA that confirmed the presence of *P. infestans* (Table 3). Mitochondrial DNA was also amplified and sequenced from the three loci in the P2, P3 and P4 regions. The Ia mtDNA haplotype was detected in the potato and tomato leaf samples tested from the same time period.

Discussion

It is hypothesized that the epidemics of late blight in England and Europe in the 1840s were started by importation of infected potato tubers, most likely from South America (de Bary, 1876; Bourke, 1964; Gomez-Alpizar *et al.*, 2007). Previous work, based on studies of diseased leaf lesions from herbaria, provide evidence of the widespread distribution of only the Ia mtDNA haplotype in England, Ireland, France

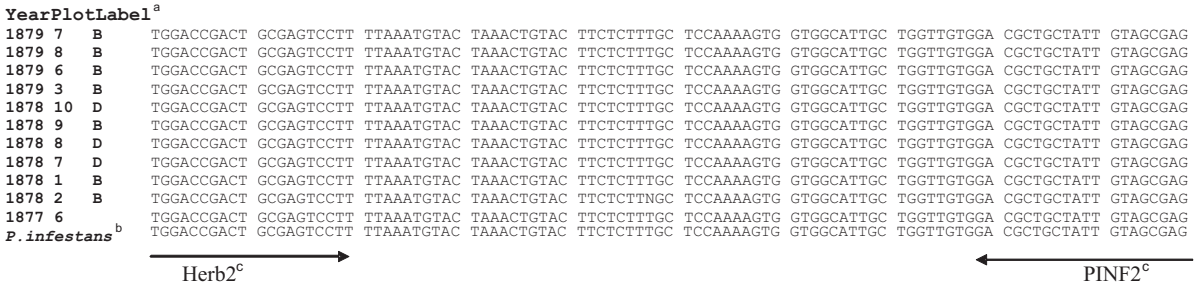

Figure 4 Internal transcribed spacer sequences (ITS) from *Phytophthora infestans* from 19th century potato tubers. Sequences obtained from PCR products amplified by *P. infestans*-specific primers (PINF2/Herb2) from one modern and nineteen 19th century (1876–1879) diseased potato tubers that were dried, ground and stored in the archive at Rothamsted Research, Harpenden, UK. (a) Samples of dried ground potato tubers were labelled D = diseased or B = bad. (b) *P. infestans* modern isolate EC3422 GenBank accession number AY770739. (c) PCR primers used to amplify DNA from the ITS2 region.

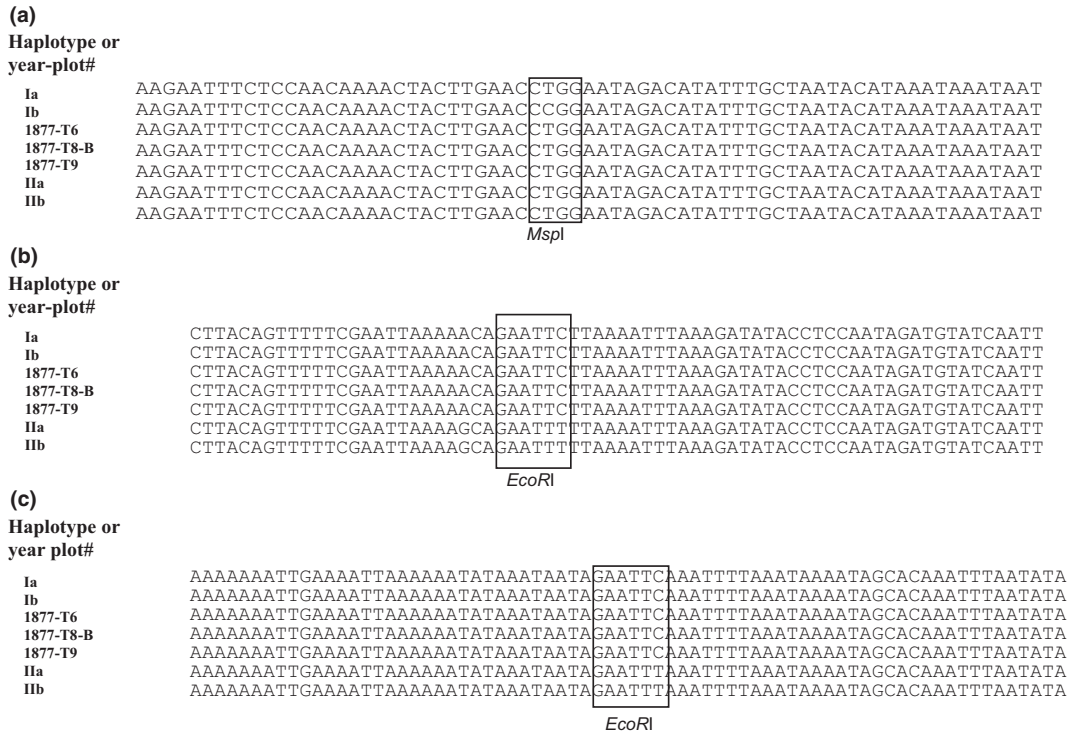

Figure 5 Mitochondrial DNA sequences from *Phytophthora infestans* from 19th century potato tubers. DNA sequences obtained from PCR amplification of three loci including (a) P2 (*nad4* gene), (b) P3 (*rpl5* gene) and (c) P4 (*cox1* gene) regions of *P. infestans* mitochondrial genome. Data shown from four modern mtDNA haplotypes (Ia,Ib,IIa,IIb) of *P. infestans* and three archive samples of diseased potato tubers from 1877. *Msp*I and *Eco*RI restriction sites are highlighted.

and elsewhere in Europe in the 1840s (Ristaino *et al.*, 2001; May & Ristaino, 2004). The detection of the Ia mtDNA haplotype of *P. infestans* in three archived tubers from the 1877 Rothamsted experiment provides the earliest evidence of the involvement of diseased tubers in the survival of the same mitochondrial lineage of the pathogen in the UK between cropping seasons. The pathogen survived between cropping seasons in tubers in some of the Rothamsted plots as indicated by real-time PCR detection of the pathogen in tubers each year (Table 2).

The Ib mtDNA haplotype of *P. infestans* was not identified in any 19th century potato tubers in this or previous studies from leaves from archival samples (May & Ristaino, 2004). In this study, mtDNA could only be amplified from three of the ground Rothamsted tuber samples, so the possibility that other mtDNA lineages were present in tubers after the 1870s as well cannot be excluded. It is known that by this time, tubers were being imported into the UK and elsewhere from both South America and Mexico (de Bary, 1876). There is clear evidence in other work with herbarium leaves that the Ia mtDNA haplotype caused epidemics during the 19th century in the UK. The Ib mtDNA haplotype of the pathogen was present by the mid-20th century in leaf samples from Ecuador, Bolivia, Asia, USA, Canada and China (May & Ristaino, 2004; Ristaino & Hu, 2009; Guo *et al.*, 2010; Ristaino, 2012). This suggests a later spread of the Ib mitochondrial lineage from the Andean region (May & Ristaino, 2004). The Ib lineage has not been detected in any samples from the UK or Europe in the 19th or early 20th century, so it is difficult to determine a time of introduction of this lineage into Europe (May & Ristaino, 2000; Ristaino, 2012). Gavino & Fry (2002) reported the Ib lineage in the Netherlands, Poland and the UK in 2002 and Griffith & Shaw (1998) reported Ib haplotypes in the UK in the 1970s.

Work with the Rothamsted archival tuber samples was not straightforward. As whole tubers were infected, dried and then ground, degradation of DNA occurred and the overall rate of success by both conventional and quantitative PCR was much lower than with samples from infected dried leaf lesions. Levels of DNA obtained from infected Rothamsted tubers as determined by real-time PCR was lower than from infected dried leaf lesions, suggesting degradation of DNA in the ground archival materials. Others have used real-time PCR to detect and quantify the pathogen *in vivo* in infected living tubers and relative amounts of pathogen DNA is much higher in tubers from those studies (Atallah & Stevenson, 2006; Llorente *et al.*, 2010).

The introduction, establishment and spread of *P. infestans* outside its native range is a classic example of a biological invasion by a plant microbe (Gomez-Alpizar *et al.*, 2007; Brasier, 2008). The spread of new clonal lineages of the pathogen in potato tubers distributed from Mexico to North America and into Europe has occurred in recent times, and from Russia to North China (Fry, 2008; Guo *et al.*, 2010; Ristaino & Hu, 2009). The pathogen is still a serious threat to worldwide potato production and the Ia mtDNA haplotype is still widespread (Cooke *et al.*, 2006; Ristaino & Hu, 2009), although greater mtDNA haplotype diversity and more aggressive strains are now found in many areas of the world (Cooke *et al.*, 2006; Gomez-Alpizar *et al.*, 2007; Fry, 2008; Hu *et al.*, 2012).

Lawes and Gilbert planted the blight resistant cultivar Champion in the Hoosfield experiment from 1880 and the blight susceptible cv. Rock from 1876 to 1879 (Gilbert, 1888, 1900; Glendinning, 1983). Samples of diseased tubers were less common in the archives after the blight resistant cultivar was planted. Although resistance (*R*) genes have been deployed over many years in the UK, development of stable resistance to the pathogen is still a moving target. Populations of *P. infestans* with low pathogen genotype diversity within a single clonal lineage may have high virulence diversity (Fry, 2008; Guo *et al.*, 2009). There is considerable interest in identifying the evolution and function of pathogen effector genes and their corresponding host *R* genes in potato and tomato (Huang *et al.*, 2005; Whisson *et al.*, 2007; Jiang *et al.*, 2008).

The quantitative PCR assay developed here should be a useful tool in plant disease diagnostic clinics to test seed potatoes or tomato transplants for the presence of late blight. The PCR assay was optimized and the standard curve allowed quantification of DNA abundance directly in fresh and dried tissue. The specificity of the PCR assay for other tuber-borne *Phytophthora* species was also tested. This assay could be used to evaluate the colonization of host tissue by the pathogen and could also be used to screen germplasm for resistance to the disease. The quantitative PCR assay could be used to select germplasm that reduces the colonization potential of the pathogen in host tissue. A similar quantitative PCR assay was developed for *Peronospora tabacina* and was used to differentiate susceptible and resistant tobacco germplasm using time course experiments (Blanco & Ristaino, 2011).

Late blight is still a serious disease problem in organic potato production (Leifert & Wilcockson, 2005; Finckh *et al.*, 2006; Hu *et al.*, 2012). The pathogen has re-emerged as a significant disease threat to the organic tomato industry in the US where management options are limited (Stone, 2009). Few organic pesticides are available for disease control. Some European countries have banned the use of copper for control of late blight in organic farming (Dorn *et al.*, 2007; Hospers-Brands *et al.*, 2008) and there is once again renewed interest in testing the effect of both animal manures and inorganic nitrogenous fertilizers on tuber blight (Finckh *et al.*, 2006). Soil fertility amendments can have a large effect on potato yield. Finckh *et al.* (2006) documented that only 30% of the variation in yield of potato is due to reduction of late blight. Temperature, growth duration and soil mineral N in combination with control of late blight explained 75% of the observed variation in yield.

The increase in crop foliage in well-fertilized plots and early increases in haulm production probably led to tuber bulking and increased pathogen inoculum load on leaves that subsequently infected tubers later in the cropping season. Thus plots with increased yield also had a higher proportion of blighted tubers (Table 1). Soil fertility amendments, including the addition of N and combinations of

inorganic minerals, increased potato yield but also increased the incidence of tuber blight in the Hoosfield experiment. This effect of early tuber bulking on potato yield has been observed in modern experiments on organic fertility amendments and late blight (Hospers-Brands et al., 2008). The proportion of tubers diseased is likely to have been underestimated in the Hoosfield study because some diseased tubers may have rotted before harvest.

The early experiments at Rothamsted may have been the first specifically designed to investigate whether fertility amendments could affect the severity of a plant disease. The results of this 19th century trial (Gilbert, 1888, 1900; Hall, 1905) are still relevant in the current debate about the use of inorganic or organic fertilizers and pesticides for control of late blight (Stone, 2009).

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Supporting Information

Additional Supporting Information may be found in the online version of the article:

Sequence data has been deposited into GenBank. Accession numbers are: mitochondrial P2 regions FJ785405, FJ785406, FJ785407; P3 regions FJ786021, FJ786022, FJ786023; P4 region FJ786024, FJ786025, FJ786026.

Table S1 Average yield (t ha^{-1}) of potatoes in experiments to study the effects of fertilizer treatment on yield and severity of late blight disease (caused by *Phytophthora infestans*) at Rothamsted, Harpenden, UK for the period from 1876 to 1880. At harvest, in September/October, potatoes were separated into good, small and diseased tubers.

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